AD_________________

Award Number: DAMD17-02-1-0177

TITLE: Non-Immunogenic Structurally and Biologically Intact Tissue Matrix Grafts for the Immediate Repair of Ballistic-Induced Vascular and Nerve Tissue Injury in Combat Casualty Care

PRINCIPAL INVESTIGATOR: Nathaniel Bachrach, Ph.D.

CONTRACTING ORGANIZATION: LifeCell Corporation
Branchburg, New Jersey 08876

REPORT DATE: December 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
December 2003

3. REPORT TYPE AND DATES COVERED
Annual (15 Nov 2002 - 14 Nov 2003)

4. TITLE AND SUBTITLE
Non-Immunogenic Structurally and Biologically Intact Tissue Matrix Grafts for the Immediate Repair of Ballistic-Induced Vascular and Nerve Tissue Injury in Combat Casualty Care

5. FUNDING NUMBERS
DAMD17-02-1-0177

6. AUTHOR(S)
Nathaniel Bachrach, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
LifeCell Corporation
Branchburg, New Jersey 08876

E-Mail: nbachrach@lifecell.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
In the first year of this graft processes were developed that provided a decellularized, cryopreserved, and freeze-dried human umbilical artery and vein for use as off-the-shelf vascular grafts. Short term animal studies indicated that the vein graft had potential as a hemodialysis graft. However, close evaluation of those tissues indicated that there were defects that may have clinical impact. This past year the source of he defects was determined to be the freeze-drying process. Ongoing efforts toward process optimization and design modifications that will provide undamaged tissue grafts are presented in this report. Essential processing modifications, procurement systems and a total quality plan were also developed and implemented. Biocompatibility testing was performed and when it was appropriate biomechanical verification testing was completed. In addition, the umbilical artery was evaluated for the first time in large animals. Graft related adverse events led to further tissue processing optimization. That work is presented in this report but artery process development is still ongoing.

14. SUBJECT TERMS
Vascular graft, freeze-dried, umbilical, biological activity, repopulation, patency, arterio-fenous shunt, nerve graft

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

I. Introduction:

The purpose of this project is to develop the processes to procure umbilical vascular tissue, process from them suitable vascular conduits to safely serve as off-the-shelf vascular graft prostheses for below-the-knee vessel replacement, AV access, and other clinically relevant applications. Previously methods had been developed for dissection and decellularization of the tissues and biomechanical characterization had been completed. Some animal studies had demonstrated host cell repopulation and the potential for re-endothelializaion of the vein tissues in particular. This report focuses on optimization of a number of systems fundamental to the successful processing of the grafts and tissue procurement. This includes the traceable and controlled tissue procurement, quality plan, and a safety holding step. In addition, some characterization such as biocompatibility, transmission electron microscopy and multi-puncture mechanical testing was completed. Additional process optimization with respect to freeze-drying is revisited due to the presence of lumenal cracks found in some tissue grafts. An in vivo study with the umbilical artery as an interpositional carotid graft in a porcine model is presented. The artery was not successful in this model and studies are ongoing to determine the cause of failure and determine if the source lies in the processing of the artery tissue.

Objective 1:
Optimize procurement and processing protocols for umbilical vessels to obtain acellular grafts that retain matrix integrity and biological activity.

Procurement

In the past LifeCell had attempted to access umbilical cord tissue from a variety of different types of agencies, including umbilical cord stem cell programs, tissue banks, and hospital obstetrical units. Requirements included procurement of cords immediately after birth placement in RPMI 1640[1] solution with 300 mg/L L-glutamine and 10 mg/L Gentamicin. The umbilical cords were to be stored at 4°C until time of shipment and maintained on wet ice during shipment. Tissue would be received at LifeCell within 30 hours of birth and would not contain any holes, tears, or clamp marks.

For pre-clinical studies LifeCell established a procurement arrangement with the National Disease Research Interchange (NDRI). NDRI is a contract tissue bank organization located in Philadelphia which provides human tissues for research purposes. This arrangement does not provide the level of control necessary for procurement of tissues for clinical use, nor does NDRI have the ability to provide serological and other donor information essential to obtaining medical director approval for human tissue clinical use. While research has continued with umbilical tissue procured through NDRI LifeCell has independently developed methods for clinically acceptable tissue procurement from Saint Francis Hispital (SFH) of Tulsa Oaklahoma.

Procurement Procedure

LifeCell Corporation receives umbilical cords from Saint Francis Hospital, Department of Labor and Delivery, located in Tulsa, Oaklahoma. The SFH internal review and ethics board (IREB) has approved a clinical procurement protocol and informed consent for obtaining the umbilical tissue. The donor is deidentified from LifeCell and SFH has obtained a certificate of confidentiality from the NIH to protect the donor patients' privacy. Consent includes the provision for research use as required by tissue banking standards in order to use the tissue for this clinical research project. All nurses and physicians involved in the collection of research data are trained as required by the IREB at SFH. No umbilical cords or other information is obtained from expectant mothers without a signed informed consent form.

The nurse or physician obtains patient's consent at time of admission if initial donor criteria are met. The nurse or physician will then complete the patient assessment questionnaire and assign a patient donor number for use in all documentation that will be seen by LifeCell Corporation. The patient assessment questionnaire includes questions regarding medical and social history and is designed to determine evidence of high risk behavior as defined by the Center for Disease Control.

Following delivery, the cord is cut and the placenta is delivered with as little traction as possible. The cord is then clamped proximally in a way that maximizes usable tissue length and cut from the placenta using a scalpel. Blood is then allowed to drain from the cord and the cord is placed in a sterile container with saline. The cord is then transported to the pathology laboratory on ice for detailed assessment to previously defined rejection criteria.

The Umbilical Cord Data Form is then filled out, in part by labor and delivery and in part by the placentologist. This data form documents the informed consent, the cord dimensions, some basic donor information (mother and child), a physical assessment of the mother for risk factors, and the pathologist's gross inspection of the tissue.

---

[1] RPMI-1640 is a standard commercially available solution formulation developed for organ transport.

Processing

Holding Step Shift

Since procurement of tissue is variable and graft processing should lend itself to convenience and scalability, a holding step, was introduced into the vascular graft process. Previously, tissues were harvested fresh and processing began immediately. Since infant births are variable, neither the time nor the quantity of umbilical cord availability is predictable. With the addition of a holding step, cords could be collected and frozen until an appropriate lot size has accumulated. Then the lot can be processed at a convenient time.

Currently, umbilical veins and arteries enter the holding step after dissection. The umbilical vein is filled with dyed cryoprotectant, ligated, dissected free from the surrounding tissue, and then submerged and incubated in cryoprotectant solution at 4°C overnight with rotated mixing. Following incubation, the vein and arteries are removed from the surrounding cryoprotectant solution and frozen at -80°C in new containers. Vessels can then be held at this deep-freeze step for a prolonged period of time prior to processing.

A viable manufacturing process must allow a hold before any value added processing of the umbilical cords to provide time for receipt and review of all donor information and evaluation of presence of pathogens. To achieve this, the holding step described above has been shifted to occur prior to umbilical cord dissection. In the new process the umbilical vein is filled with cryoprotectant and ligated as before. The entire cord is then submerged in cryoprotectant and incubated overnight at 4°C with rotation and frozen the next day. This process change could only be achieved after determination of cryoprotectant penetration into the tissues through the thick amnionic membrane and layer of Wharton's Jelly. A transmission electron microscopy study has shown that compared with fresh tissue, the tissue processed after the whole cord holding step retains the basic essential microstructural elements of the tissue while eliminating the potential for cell mediated responses to the tissue when implanted (Appendix A). A study was also performed to evaluate the thermal properties of the tissue as a measure of effective cryoprotectatnt incubation (Appendix B). The study concluded that the vessels in the umbilical cord are cryoprotected to the same extent as dissected tissue processed by current methods.

Lumenal defects in the freeze-dried umbilical vein grafts

In the last report a short term pre-clinical feasibility study was described. The study was used to evaluate the porcine AV-shunt system for use as a chronic implant model. A final pathology report completed later on that study found that one graft had significant hemorrhagic dissection of the media. These findings suggest that the structural integrity of the umbilical vein graft (UVG) had been compromised. The root cause of this breech in structural integrity was investigated and two distinct causes were found to have contributed to this failure:

- Defects were detected on the lumenal surface of that explanted graft. Gross in vitro evaluation of the lumenal surface of freeze dried UVGs as produced for this experiment revealed occasional crevices. This damage has been demonstrated to result from the particular freeze-drying process used, and is a directly assignable cause to this mode of failure in the animal model.

- Histopathology of the explanted grafts revealed a cross-species immune response. This kind of response typically includes rapid resorption of extracellular matrix components responsible for a blood vessel's mechanical integrity. Therefore, an accelerated loss of structural integrity is a predictable consequence of implanting intact human tissue in an animal model.

Options for Process Optimization

A series of experiments have been performed that demonstrate the source of the cracking of the tissue is the final stages of the freeze-drying process. Currently the only way to provide 100% assurance that vessels do not contain lumenal cracks is to provide a frozen final graft that is thawed at the point of use.

Storing the graft frozen instead of freeze drying represents a significant process change. Firstly the graft had not been frozen below -35°C in the freeze-drying process and now the graft would be held at -80°C. Secondly, the new thawing process without the vacuum provides a different environment for the tissue and has the potential to cause tissue damage. Verification testing was performed on the graft including burst testing, suture retention, and multipuncture mechanics (which is relevant for hemodialysis grafts and can provide hints of more subtle tissue damage for other grafts as well). A full description of the biomechanical testing and failure analysis that were performed is attached as Appendix C.

Other Strategies to Alleviate Tissue Damage

While these results demonstrate that the frozen-thawed UVG is an acceptable graft it is not the optimal process for combat casualty care. Controlled storage and transport of the graft at -80°C is not a simple matter. A more optimal solution is being evaluated in ongoing studies.

The most straightforward option is to provide a freeze-dried graft that is not dried to the level where cracks are initiated in the tissue. This solution would be viable only if the threshold residual moisture level in the tissue where cracks initiate could be identified. Then adjacent (or satellite) samples of tissue would be evaluated as a quality measure to ascertain that the drying process did not go to far for any given umbilical vein (given the biological variation between donor tissues). Exploring this design option required the following:

1. Development of a robust and accurate method for determining residual moisture levels in umbilical tissue samples
2. Evaluation of the within graft residual moisture variation to determine the limitations on residual moisture for an adjeacent section of tissue.
3. Determination that the satellite sample was indeed representative of the residual moisture in the whole vein segment.
4. Determine the nominal residual moisture threshold where lumenal cracks begin to form in the tissue.

A thermogravimetric method has been developed for determining residual moisture levels in umbilical tissue samples. This method was then applied to evaluate the within and between graft variability, and to compare the satellite samples to those values. It turns out that the variability within a graft is higher than the variability between donor vein grafts. Also, it was determined that satellite samples do not provide a representative measure of the residual moisture in the whole vein segment (Appendix D). These results led to the following conclusions.

1. Freeze-drying to a higher residual moisture level is not an option as there is no way at this time (without 100% destructive testing) to achieve a sufficient assurance level that any given vessel does contain a segment that was dried too much and has developed a lumenal crack.
2. This level of variability in the drying of the tissue is an indication that the drying process has not been sufficiently optimized for this particular complex tissue.

These conclusions in turn have led to a series of ongoing experiments that are designed to provide uniform drying of the tissue.

In addition to this effort at freeze-drying optimization there are other avenues that are being explored. The objective is to provide a tissue graft that is an immediate-care option: an off-the-shelf graft with minimal storage requirements and very little preparation time. While freeze-dried grafts provide long shelf lives and not very restrictive storage conditions the rehydration of the

vessels can take over an hour. Another process optimization that is being explored is to provide a graft that can be prepared in much less time.

Biocompatibility

Processing protocols have been designed to achieve biocompatible vessels that would be acceptable for the intended use - clinical implantation. The umbilical artery process has not yet been optimized (see section on artery processing) so only the umbilical vein graft (UVG) has been tested to date. Table 1 provides summarized the biocompatibility testing that has been competed on the UVG. A more detailed review of the tests and their results is provided in Appendix E.

Table 1: Summary of biocompatibility tests completed testing for the UVG

Test	**Test Method**	**Test Article**	**Result**
Cytotoxicity	<u>Test system:</u> in vitro L929 mouse fibroblasts, 48hr @ 37°C • Elution: 2gm tissue @ 37°C for 24 hr in 10ml applied to test system • Agar Diffusion: lumenal surface applied to 0.5-2g/ml agar layer on test system	Frozen UVG	Not cytotoxic
Hemolysis	<u>Test system:</u> human red blood cell concentrate • Direct contact:0.1gm applied directly to test system • Extracted:0.1g tissue @ 37°C for 24 hr in 1.5ml saline applied directly to test system	Frozen UVG	Not hemolytic
Pyrogenicity	<u>Test system</u>: White rabbits' febrile response • Extraction: 4g tissue @ 37°C for 24 hr in 80ml saline	Frozen UVG	Not pyrogenic

Quality Plan

As a fundamental part of processing optimization in the development of a graft that will have use in combat casualty care it is essential that it be developed with a robust quality plan. Part of the necessity of this plan is a quality assurance level even on development grafts used for in vitro and in vivo testing that will be reliable. This is critical in light of the graft related events that have been found in those in vivo studies and uncertainty regarding their processing environment.

Procedures have been developed to minimize the chance of infection during UVG processing consistent with general tissue industry practices such as donor screening, microbiological testing, antibiotic and antifungal treatments, utilizing sterile processing supplies, and a controlled processing environment. The processing and storage of the tissue at the LifeCell facility conforms to 21 CFR 1270 Human Tissue Intended for Transplantation and the American

Association of Tissue Banks (AATB) Standards for Tissue Banking. The details of what is included in the plan are provided in Appendix E.

II. Objective 2:

Establish the biomechanical characteristics of these unique, biologically intact umbilical vessel grafts in failure mode analysis.

(see mechanical verification testing and failure analysis in Appendix C)

III. Objective 3:

Demonstrate that umbilical vessel grafts transplanted, as carotid interposition implants, in an animal model system maintain patency in the absence of dilatation, aneurysm formation or neointimal hyperplasia.

The 2002 annual report described the successful transplantation of the umbilical vein as an artery-vein shunt. The success reported was very promising. This year the umbilical arteries were implanted in a porcine model as carotid interposition grafts. The arteries used in that experiment were frozen-thawed grafts to avoid the recently discovered lumenal cracking that had been discovered with the vein graft. These grafts were implanted for a 7 day period to allow for relevant data to be collected without risking an immune response severe enough to compromise graft function. The grafts failed due to rupture of some kind and the animals did not survive the experiment. The details of the experiment are presented in Appendix F. It is not certain what cause the fate of these implants. However, a exhaustive evaluation of the ability to obtain substantial intact and robust sections of umbilical arteries have been performed to try and determine if the root cause of the failures lies in an inherent weakness of the tissue or one introduced during dissection. Shredding and weak areas of the arteries has been discovered in areas where gross observation does not indicate such damage (Appendix G). The effort to optimize processing for the artery to provide robust tissue for implantation is ongoing.

IV. Objective 4:

Demonstrate in an animal model that these umbilical vessel matrix grafts maintain patency in clinically relevant application simulating long length with low flow dynamics.

The 2002 annual report described the severe inflammatory response that was elicited by the umbilical vein graft when implanted in a porcine model for approximately two weeks. Other than a small foreign body reaction to the suture material, the inflammatory reaction within the grafts was consistent with a cross-species immune response. The positive results in our biocompatibility testing (described above) support this conclusion.

To further our understanding of the limitations of this animal model for the umbilical vein and artery grafts immunohistochemical staining methods were developed using CD3 as a T-Lymphocyte marker. This staining was then used to evaluate the cells present in the tissue from that study where there was a significant inflammatory response in the outer portion of the implant (Figure 1a). The immunohistochemical evaluation with CD3 provides more substantive evidence to help confirm that the response was indeed a cross-species immune mediated reaction (Figure 1b). This kind of response to the tissue is a strong indication that the matrix components are well preserved thus eliciting a cross-species reaction in the absence of the Major Histocompatibility Complex Antigens (Class I&II) or any significant cellular debris or remnants. This is a positive result in the sense that the vessels are more likely to achieve the desired effect of repopulation, revascularization and endothelialization and provide long term patency as a clinically useful graft.

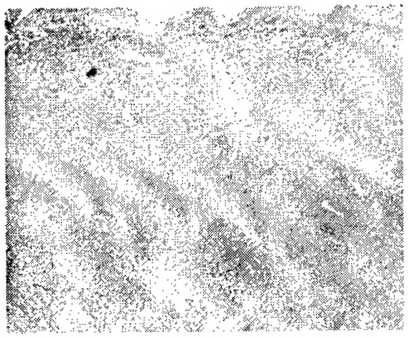
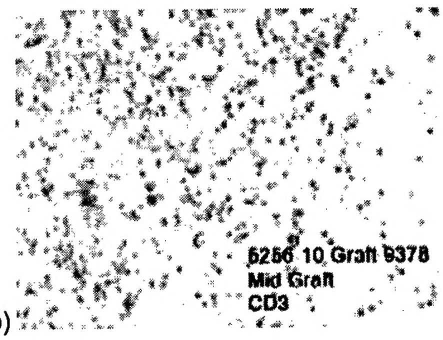
(a)  (b) 

Figure 1: Host tissue inflammatory response along graft/native tissue junction is shown on H&E staining (a) and with CD3, a T-Lymphocyte marker, indicating immune mediated response.

V. Objective 5:
Optimize procurement and processing protocols for nerve tissue to obtain an acellular graft that retains matrix integrity and biological activity.

No further progress has been made this past year toward Objective 5.

VI. Objective 6:
Demonstrate repopulation of these unique, biologically intact nerve matrix grafts and restoration of neural function in a clinically relevant nerve defect model system.

No further progress has been made this past year toward Objective 6.

VII. Key Research Accomplishments:

- Established source and methods for procurement of umbilical cords suitable for clinical use.
- Instituted a safety holding step during which umbilical cords can vessel grafts can be frozen until a convenient processing time is reached.
- Demonstrated that the processed veins are still microstructurally intact even at the electron microscopy level.
- Established the biomechanical profile for frozen vessels and their suitability for clinical use.
- Established the biocompatibility of the vein grafts
- Established the response to the umbilical vein graft in the porcine model was immune-mediated.
- Determined root cause of potential failures of the vein graft and made progress toward solutions for optimizing processing.
- Developed quality plan, including processing controls and tissue evaluation for the controlled processing of umbilical tissues.

VIII. Reportable Outcomes:

N/A

IX. Conclusions:

The production of decellularized and freeze-dried vascular grafts from human umbilical vessels was accomplished while substantially maintaining tissue microstructure biological activity and biomechanical properties. It is expected that umbilical vein and artery grafts implanted in a long-term clinically would demonstrate patency as well as significant repopulation and remodeling by the surrounding host tissue. Such a graft would be a desirable alternative to current interventions for hemodialysis access; occluding non-remodeling synthetic PTFE grafts and inconsistent native fistulas, below-the-knee vessel replacement; amputation, and coronary artery bypass grafting.

The necessary systems have been developed for procurement and quality systems as part of robust processing to provide this graft in a clinical setting. There is still design optimization underway in terms of successful freeze-drying of the vessel that will provide a practical storage condition for these grafts in addition to their shelf lives. This addition is an ongoing objective for this project. Also, as adverse events have occurred clinically it is becoming an essential design aspect for aseptically processed tissues to provide some level of microbial reduction. Exploration into the efficacy of such treatments and their impact on the maintenance of the tissue biomechanical integrity and biological activities are also ongoing objectives for the coming project year.

The progress made this past year and anticipated for the coming year should provide a substantial basis for the advancement of these processed vascular tissue grafts as candidate grafts for vascular combat casualty care.

X. References:

Bergman, R.A., et al. *Atlas of Microscopic Anantomy: Section 13 – Female Reproductive System, Plate 13.261 Umbilical Cord.* Virtual Hospital, University of Iowa Health Care. 1992 – 2003.

ISO 7198:1998(E) "Cardiovascular Implants – Tubular vascular prostheses

Nanaev, A.K. et al. *Stromal Differentiation and Architecture of the Human Umbilical Cord.* Placenta (1997), 18, 53-64.

Appendix A: Transmission Electron Microscopy of Vein Grafts

Objective

The objective of this electron microscopy study was to evaluate the effects of chemical decellularization and freeze-thaw of the tissue. Processed and unprocessed umbilical vein tissue was compared by electron microscopy to determine that there is a removal or substantial destruction of cellular organelles that could provide the potential for an immunogenic response and retention of the fundamental components and structures of the tissue.

The test article in this evaluation was umbilical vein processed by standard methods (n=5 donors, longitudinal and cross section samples). The control article was umbilical vein received and cryopreserved but with no further processing (n=1 donor, longitudinal and cross section samples).

Results

The intima of the unprocessed veins consists of an endothelial layer, a basement membrane, an internal elastic lamina and collagen (Figure 1a&b) In the processed tissues the endothelial layer had successfully been removed (Figure 1c). Only very occasional cellular debris was reported.

In the control tissue the basement membrane was present and fairly continuous covering about 75% of the lumen surface and an internal elastic lamina was evident (Figure 1b). There was over 50% basement membrane coverage in all (5) veins evaluated. Three of the five veins had 70% + coverage, which is similar to that seen in the fresh samples. In some cases there were small tears suggestive of damage due to mechanical handling either as part of the tissue process or processing the tissue for electron microscopy.

Appendix A: Transmission Electron Microscopy of Vein Grafts

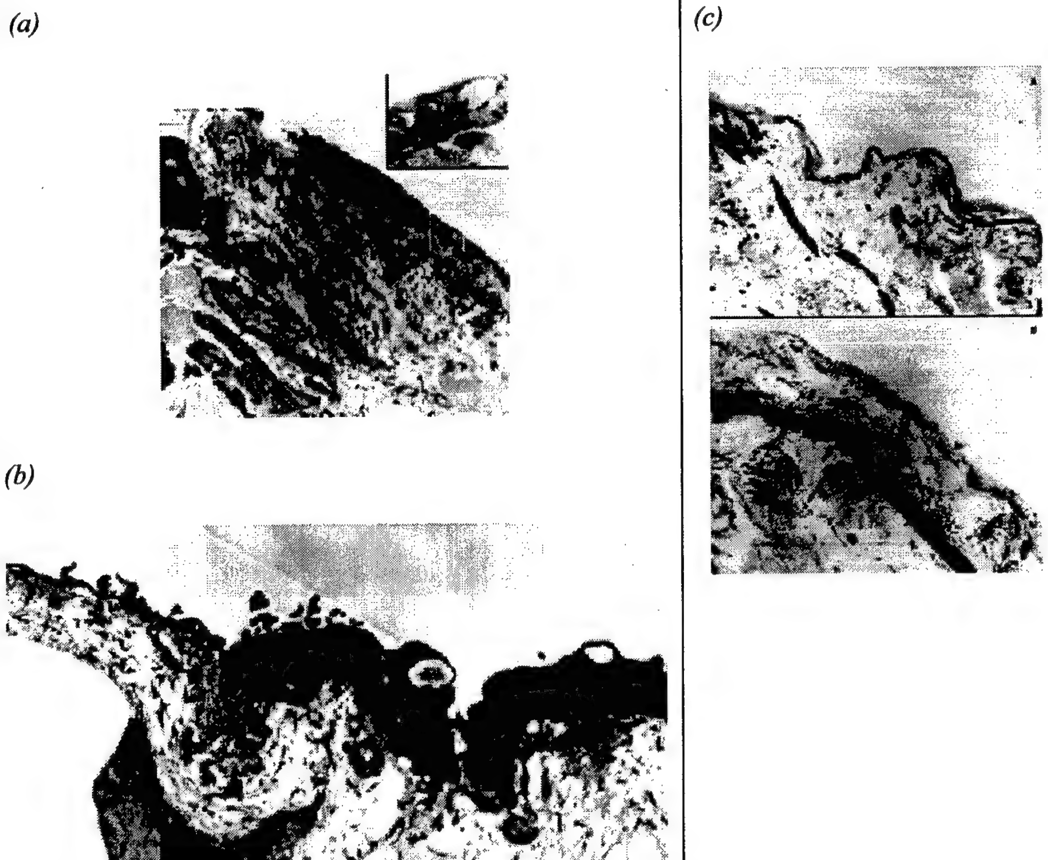


Figure 1: Unprocessed human umbilical vein has (a) intact endothelial cells (3,000x) containing a Weibel-Palade body and (b) intact, a continuous basement membrane, elastin, and collagen, (4,000x). (c) UVG has no endothelial cells but has an intact basement membrane and supporting extracellular matrix (top @ 3,000x, bottom at 10,000x)

The control tissue media was thick, compact, and rich in smooth muscle cells (SMC) that were well preserved SMC with few organelles, a central nucleus, a fine network of filaments, dense bodies, dense plaques, caveolae, and a continuous basal lamina (Figure 2a). In the processed tissue the SMC nuclei and organelles were successfully removed. No convincing cell remnants (recognizable organelles) remained (Figure 2b). The basal lamina and the filamentous network containing smooth muscle filaments, dense bodies, and dense plaques, remained. These remaining cell components have been defined as cellular debris.

Appendix A: Transmission Electron Microscopy of Vein Grafts

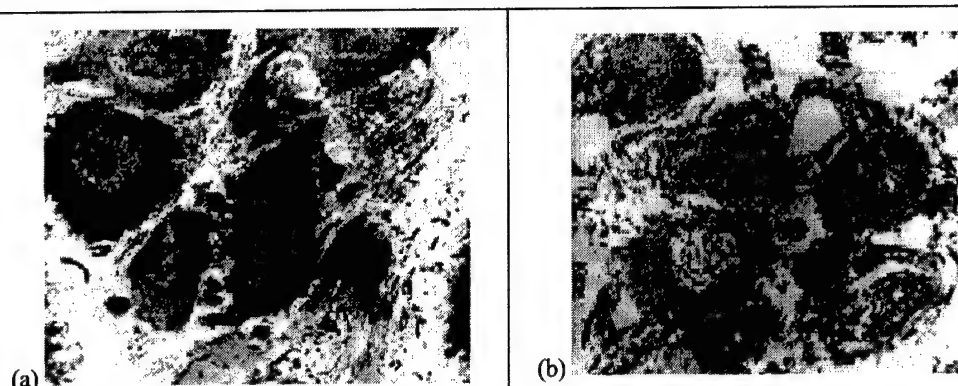


Figure 2: Smooth muscle cells of the unprocessed umbilical vein (a) show centrally located nuclei, filamentous network and cell organelles (2,500x). In the processed tissue (b) the cell organelles are unrecognizable (3,000x).

Also present in the extracellular matrix were occasional wisps of elastin, collagen, fine filaments and ground substance. Collagen periodicity was evident and maintained in processed tissue (Figure 3).

Figure 3: Lifecell processed umbilical vein. Collagen fibers in UVG demonstrating typical periodicity (35,000x).

While there is some controversy in the literature[2] (Bergman, Nanaev) regarding the presence of a adventitial layer in the vessels of the umbilical cord it was noted in our observations as a thin layer consisting primarily of collagen. The few cells seen in the adventia and in the surrounding Wharton's Jelly were substantially removed or reduced to debris.

[2] Bergman, R.A., et al. *Atlas of Microscopic Anantomy: Section 13 – Female Reproductive System, Plate 13.261 Umbilical Cord.* Virtual Hospital, University of Iowa Health Care. 1992 – 2003. and Nanaev, A.K. et al. *Stromal Differentiation and Architecture of the Human Umbilical Cord.* Placenta (1997), 18, 53-64.

Appendix A: Transmission Electron Microscopy of Vein Grafts

Conclusions

The most significant difference in tissue morphology between the fresh umbilical veins and the UVG is the successful removal of the endothelium, while maintaining a relatively intact basement membrane. Additionally, the cellular components of the media, adventitia, and Wharton's Jelly, that may have the potential to produce an immune response, were either removed or disrupted beyond recognition. These cellular components include the nucleus and nuclear components, the cell organelles, and the cell membranes (excluding the basal lamina).

Cell cytoskeletons contribute to mechanical integrity of the matrix in tissues with a high cell to tissue ratio. It is believed that the filamentous network of the cells that remains in the UVG is important in maintaining tissue structure.

Appendix B: Cryoprotection of Undissected Umbilical Vessels

Test articles:

- Freshly isolated vein and artery tissues.
- Vein and artery tissues isolated after the whole (undissected) cord treated with 50% (w/w) X-101 for 4 hours.
- Dissected vein and artery tissues treated with 50% X-101 overnight (~ 19 hours)
- Vein and artery tissues isolated after the whole (undissected) cord treated with 50% (w/w) X-101 overnight (~ 22 hours)
- Freshly isolated vein and artery tissues partially dehydrated for ~16 hours under 91% RH in a closed container.

Note: Vein and artery tissues included some adherent Wharton's jelly.

Differential scanning calorimetry (DSC) analysis was performed using the following temperature program. Each sample was cooled at 1 °C/min from 10°C to –60°C, held for 10 min at –60°C, and then warmed at 2 °C/min from –60°C to 20°C. Actual final temperature was roughly –64°C before warming.

Results:

Melting enthalpy was calculated for the various test samples. The data is presented below.

	Melting Enthalpy	
	Vein (J/g sample)	Artery (J/g sample)
Fresh tissue	257.1	268.4
Whole cord, 4-h treatment	150.1	192.4
Dissected tissue, ~19-h treatment	93.8	77.0
Whole cord, ~22-h treatment	71.2	79.7
Partially dried under 91% RH	186.3	220.5

DSC also was used to determine the onset temperatures for T_h and T_g of samples (onset temperature). The data are presented below:

	T_h (°C)		T_g (°C)	
	Vein	Artery	Vein	Artery
Fresh tissue	-14.7	-13.4	-45.0, -28.1	No sample
Whole cord, 4-h treatment	-16.3	-12.6	-23.3	-27.2
Dissected tissue, ~19-h treatment	-14.9	-18.0	-20.2	-20.7
Whole cord, ~22-h treatment	-18.9	-23.6	-21.2	-22.4
Partially dried under 91% RH	-14.5	-13.1	-36 (?)	-38 (?)

(?) not clearly identifiable on the thermograms

After DSC measurement, water content was determined by gravimetric method. The data are presented below.

	Vein (g/g dw)	Artery (g/g dw)
Fresh tissue	7.4	9.1
Whole cord, 4-h treatment	1.7	2.8
Dissected tissue, ~19-h treatment	0.8	0.7
Whole cord, ~22-h treatment	0.6	0.7
Partially dried under 91% RH	4.0	3.7

Conclusions

The data demonstrate that the vessels in the umbilical cord are cryoprotected to the same extent as dissected tissue that has been incubated for less time.

Appendix C: Biomechanics and Failure Analysis of Frozen Grafts

Three mechanical tests were performed in the evaluation of the frozen grafts as they relate to potential failure modes for the vein for use a hemodialysis graft. In each case the vessels were extracted and processed according to the current methods previously described (Annual report 2002) with exception of final freezing of the tissue at -80°C instead of Freeze-Drying. The tests described here were completed after thawing and rinsing the tissue.

Burst pressure testing

The burst pressure of umbilical veins after processing was evaluated by increasing intraluminal pressure by adding fluid to a vessel with a sealed end. The burst pressure was defined as the maximum pressure the vessel can sustain before a precipitous drop in pressure is measured. Initial burst testing on fresh umbilical vein were performed using a syringe to increase the pressure in the vein manually while pressure was monitored on an in line dial gage. The vessel remained hydrated during the procedure. Subsequently a testing apparatus was designed to provide a means for controlled inflation using a syringe pump and electronic data collection to more accurately record peak pressures. In the latter test the vessel was submerged in a water bath of phosphate buffered saline during the testing. The modified testing method provides for better accuracy and control. However, the protocol is sufficiently different that statistical comparison between the two groups is not appropriate.

Results

The vessel is capable of withstanding several times the normal physiologic peak systolic pressure of 120mmHg. Burst was not recorded on average until 856mmHg with a standard deviation of 288mmHg. The burst pressures ranged from 414mmHg to 1396mmHg (Attachment A-1.1). Results are shown in Table 1. Data from fresh tissue tested at a different time are included in the data table for comparison.

Table 1: Burst Strength of frozen thawed UVG and fresh tissue.

Sample type	Burst Pressure (mmHg)	
	Mean ± SD	Range
Fresh Umbilical Vein (n =8)	1344 ± 600	775 - 2430
Processed Frozen Vein (n=10)	856 ± 288	414 - 1396

SD = standard deviation

Failure Analysis

Graft consistency and the potential for acute aneurysm formation or rupture of the HUV graft when stored as frozen graft and implanted as an access graft is addressed using statistical methods below.

Graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The burst pressure is the best available measure of the likelihood of aneurysm formation due to arterial blood pressures sustained in the vein in vivo. The data are provided in Table 2.

Appendix C: Biomechanics and Failure Analysis of Frozen Grafts

Table 2: Range and confidence intervals for burst pressure

Lot #	Burst Pressure (mmHg)
10746	983
10890	621
10896	1086
10911	414
10917	776
10919	905
11029	1396
11353	931
11355	931
11376	517

Statistic	Resutls (mmHg)
N[3]	10
Mean	856
Lower Confidence limit -95%	650
Lower Confidence limit -99.5%	520
Min	414
Max	1396
Standard Deviation	288

AV access grafts typically do not sustain arterial pressures however is there is impeded outflow then arterial pressures can be realized. A graft with burst pressure of 120mmHg is considered for the purpose of this analysis as a graft that has a potential to fail. Based on the data provided, there are 2.56 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 0.5% that a graft will have the potential to fail in acute aneurysm at 120mmHg.

Suture Retention Strength Test[4]

This test is an evaluation of the ability to suture the graft material to the host tissue. Using a curved surgical needle, a 6-0 Prolene suture is passed through one wall of the graft 2 mm from the free edge using a custom designed fixture. The suture is placed through a location on the graft that is free of any adherent tissue to provide an accurate worst case measure of suture retention strength. The suture is pulled a few inches through the needle hole and the tissue sample is loaded into a tensile tester (Instron Model #5865, Merlin Software Version 5.31) clamping both suture ends in the top wedge grip and then clamping the within the bottom grip. Both ends of the suture are pulled in one direction at 150mm/min with the vessel held stationary. The maximum force that is reached before the suture pulls through the wall of the vessel is taken as the suture pull strength (Attachment A-1.2).

Results

The measured suture retention strength of frozen UVG was on average 3.8N with a standard deviation of 1.3N and a range of 1.8 - 5.4 N. The values all exceeded 1N which the rule of thumb in the industry for acceptable suture retention strength (Attachment A-1.1). Results are shown in Table 3. Data from fresh tissue tested at a different time using a 5-0 suture are included in the data table for comparison.

[3] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

[4] ISO 7198 Section 8.8

Appendix C: Biomechanics and Failure Analysis of Frozen Grafts

Table 3:	**Suture retention strength fresh tissue and of frozen UVG after thaw and rehydration.**	
Sample type	**Suture retention strength (N)**	
	Mean ± SD	**Range**
Fresh Umbilical Vein (n =8)	2.9 ± 1.1	1.3 - 4.4
Processed Frozen Vein (n=10)	3.8 ± 1.3	1.8 - 5.4
SD = standard deviation		

Failure analysis

Graft consistency and the potential for suture pull out during implantation of the UVG graft when stored as frozen graft and implanted as an access graft is addressed using statistical methods below.

Graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The suture retention strength is the best available measure of the likelihood of failure to achieve an suitable anastamoses using the UVG. The data are provided in Table 4.

Table 4: Range and confidence intervals for burst pressure

Lot #	Suture Retention (N)
10746	3.42
10890	2.38
10896	2.39
10911	5.14
10917	5.42
10919	4.26
11029	4.41
11353	3.42
11355	1.79
11376	5.05

Statistic	Result (N)
[5]N	10
Mean	3.77
Lower Confidence limit -95%	2.85
Lower Confidence limit -99.5%	2.27
Min	1.79
Max	5.42
Standard Deviation	1.29

A suture retention strength of 1N is considered to be sufficient to allow for transplantation of the graft without ripping or compromising the graft at the anastamoses. Based on the data provided, there are 2.15 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 1.6% that a surgeon could have difficulty with the implantation due to abnormally low suture retention strength.

Strength After Repeated Puncture[6]

If the graft is used for hemodialysis access the graft will be punctured repeatedly during its use, it is necessary for the tissue to withstand that kind of mechanical insult without forming an aneurysm. The approach taken was to evaluate the strength of ring segments of the LifeCelll UVG when pulled in uniaxial tension to failure (Attachment A-1.2). The tests were performed on

[5] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

[6] ISO 7198 Section 8.3.4

segments containing 24 holes/cm2 (equivalent to 18 months of clinical use based on ISO 7198). This test is designed for synthetic grafts that do not heal and therefore never get stronger. Applying this test to a tissue graft that is designed to heal in, get stronger and remodel and repair needle punctures was used to evaluate a worst case scenario and is really only relevant in the first few weeks of use.

Results

In these tests the focus was on the strength and extension of the vessel wall after puncture. The failure diameters were on average 10.5mm with standard deviation of 0.7mm and a range of 9.9 - 12.1mm. The failure tension was 5.7N/cm on average with a standard deviation of 1.3N/cm and a range of 4.1 - 8.0N/cm. The data show that the strength of punctured grafts on average is greater than 3 times the normal physiologic pressures and failed at non-aneurysmal diameters (Attachment A-1.1). The results are shown in Table 5.

Table 5: Strength after repeated puncture of frozen UVG.

N=10	Mean ± SD	Range
Failure tension (N/cm)	5.7 ± 1.3	4.1 - 8.0
Internal diameter at failure (mm)	10.5 ± 0.7	9.9 - 12.1
Ratio of tension at failure to physiologic tension (120mmHg)*	3.4 ± 0.6	2.6 - 4.2

SD = standard deviation

*The calculation is Ratio= failure tension [N/m] ÷ physiologic tension [N/m],
where failure tension [N/m] = failure tension [N/cm]*100[cm/m] and
physiologic tension [N/m] = 120[mmHg]*133.3[Pa/mmHg]*(Diameter at failure[mm]*0.001[m/mm])}

Failure Analysis

Graft consistency and the potential for repeated puncture-induced aneurysm formation or rupture of the HUV graft when stored as frozen graft is addressed using statistical methods below.
With respect to repeated puncture testing graft consistency can be defined statistically as the variance associated with the sample of grafts tested in the verification activity. The identified requirement for the multi-puncture assessment is that after simulated 18 months of simulated clinical use as per ISO 7198 that the strength of the graft is not compromised (i.e. below physiological requirements).
For the current frozen configuration the strength testing was performed on ring specimens and their diameters were used to calculate the ratio of the failure strength of the punctured graft wall relative to the calculated equivalent physiologic wall tension[7]. This parameter is the best available measure of the likelihood of aneurysm formation due to excessive puncture of a graft without accounting for the tissue's healing response. The data are provided in Table 6.

---

[7] The calculation is Ratio= failure tension [N/m] ÷ physiologic tension [N/m],
where failure tension [N/m] = failure tension [N/cm]*100[cm/m] and
physiologic tension [N/m] = 120[mmHg]*133.3[Pa/mmHg]*(Diameter at failure[mm]*0.001[m/mm])}

Appendix C: Biomechanics and Failure Analysis of Frozen Grafts

Table 6: Range and confidence intervals for multi-puncture graft failure

Lot #	Ratio of tension at failure to tension at 120mmHg
10746	**3.09**
10890	**2.62**
10896	**4.18**
10911	**3.53**
10917	**2.65**
11029	**3.76**
11353	**4.15**
10746	**3.09**

Statistic	Result (N)
[8]N	8
Mean	3.38
Lower Confidence limit -95%	2.86
Lower Confidence limit -99.5%	2.5
Min	2.62
Max	4.18
Standard Deviation	0.62

A ratio of 1 is considered to have the potential to fail and there are 3.84 standard deviations from the mean to that lower limit. Assuming a normal distribution remains even at these extremes of the distribution this translates into a probability of 0.006% that a graft will fail due to multi-puncture alone.

[8] Each graft tested was from a unique donor. In most cases 2-3 samples from the same graft were tested the mean value was included as n=1 in the statistical analysis of the population

Appendix D: Freeze Drying Variability

Motivation/Background

Full length vascular grafts can not be subjected to destructive residual moisture (RM) testing, so small satellite samples of ~1cm are taken and tested to determine the RM of the graft. Once the satellite sample is removed from the graft it is packaged and freeze-dried separately. It is currently unknown how well the satellite sample represents the RM of the graft.

Low residual moisture has been linked to lumenal cracks found in freeze-dried vascular grafts. A better understanding of residual moisture variation within a single graft should help determine the level of moisture required to produce grafts free of lumenal cracks.

Objective(s)

- To examine the residual moisture (RM) variation along a freeze-dried processed vascular graft.
- To establish that the satellite samples are an acceptable representation of the graft's RM so that it will provide a means to detect when there is an unacceptable probability that cracks may occur.
- To determine the level of RM at which cracks begin to occur and obtain a range of RM values that are unacceptable.

Results

Chart 1) Top shelf Residual Moisture measurements whole cord

Sample ID	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
11016 A	0.2622	0.2092	20.2%
11016 B	0.2525	0.1885	25.3%
11016 C	0.2638	0.2122	19.6%
11016 D	0.2181	0.1802	17.4%
11016 E	0.2696	0.1917	28.9%
11016 F	0.2789	0.2143	23.2%
11016 G	0.2973	0.2458	17.3%
11016 H	0.2764	0.2326	15.8%
11016 I	0.1933	0.1475	23.7%
11016 J	0.2023	0.181	10.5%

Range 10.5%-28.9% Mean 20.2 % Standard Deviation 5.3% Coefficient of variation 26.2%

Chart 2) Top shelf Residual Moisture measurements satellite samples

Sample ID	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
11016 S1	0.714	0.6222	12.9%
11016 S2	0.6897	0.607	12.0%
10966 S1	0.2999	0.2793	6.9%
10966 S2	0.2608	0.2481	4.9%
10932 S1	0.2728	0.2567	5.9%
10932 S2	0.4013	0.3614	9.9%
11931 S1	0.3696	0.3261	11.8%
11931 S2	0.2213	0.2025	8.5%

Range 4.9%-12.9% Mean 9.1% Standard Deviation 2.8% Coefficient of variation 31.1%

Appendix D: Freeze Drying Variability

Chart 3) Middle shelf Residual Moisture measurements whole cord

Sample ID	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
10646 A	0.3375	0.2995	11.3%
10646 B	0.2918	0.2477	15.1%
10646 C	0.3097	0.2673	13.7%
10646 D	0.4358	0.3597	17.5%
10646 E	0.2949	0.2512	14.8%
10646 F	0.3429	0.2826	17.6%
10646 G	0.4293	0.3534	17.7%
10646 H	0.4581	0.3942	13.9%
10646 I	0.3407	0.2782	18.3%
10646 J	0.5365	0.4408	17.8%

Range 11.3%-17.8% Mean 15.8% Standard Deviation 2.4% Coefficient of Variation 14.9%

Chart 4) Middle shelf Residual Moisture measurements satellite samples

Sample ID	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
10646 S1	0.2556	0.2328	8.9%
10646 S2	0.2862	0.2643	7.7%
10961 S1	0.4891	0.4461	8.8%
10961 S2	0.4828	0.446	7.6%
10929 S1	0.2581	0.2446	5.2%
10929 S2	0.2034	0.1898	6.7%
11922 S1	0.5189	0.4693	9.6%
11922 S2	0.4659	0.4204	9.8%

Range 5.2%-9.8% Mean 8.0% Standard Deviation 1.5% Coefficient of Variation 19.1%

Chart 5) Bottom shelf Residual Moisture measurements whole cord

Sample ID	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
11347 A	0.4751	0.4322	9.0%
11347 B	0.6973	0.6087	12.7%
11347 C	0.8233	0.7298	11.4%
11347 D	0.2381	0.222	6.8%
11347 E	0.399	0.3656	8.4%
11347 F	0.2915	0.2684	7.9%
11347 G	0.5365	0.4802	10.5%
11347 H	0.4491	0.4083	9.1%
11347 I	0.5974	0.529	11.4%
11347 J	0.5294	0.4666	11.9%

Mean 9.9% Range 6.8%-12.7% Standard Deviation 1.9% Coefficient of Variation 19.7%

Appendix D: Freeze Drying Variability

Chart 6) Bottom shelf Residual Moisture measurements satellite samples

RM	Initial Wt of Tissue (g)	Tissue Wt after 24 hours (g)	RM
11347 S1	0.2307	0.2225	3.6%
11347 S2	0.4187	0.3897	6.9%
11846 S1	0.3666	0.3431	6.4%
11846 S2	0.3945	0.3604	8.6%
11105 S1	0.5297	0.4793	9.5%
11105 S2	0.4263	0.3903	8.4%
11921 S1	0.6724	0.5941	11.6%
11921 S2	0.5978	0.5254	12.1%

Range 3.6%-12.1% Mean 8.4% Standard Deviation 2.8% Coefficient of variation 33.1%

Chart 7) Summary of the Statistical Test and the Significance
Alpha=0.05; IF <0.05 Significantly different

	Whole to Satellite			Satellite only		Whole only	
	Top shelf	Middle shelf	Bottom shelf	Top to Middle shelf	Middle to bottom shelf	Top to Middle shelf	Middle to bottom shelf
T-test	0.0021	0.0001	0.2691	0.5346	0.8198	0.0267	0.0000
	Different	Different	Similar	Similar	Similar	Different	Different
F-test	0.1647	0.2588	0.1790	0.1712	0.1773	0.0122	0.2887
	Similar	Similar	Similar	Similar	Similar	Different	Similar

Chart8) Sample Cracking information

	Location	Rehydrated length	Number of Cracks	Location of Cracks	Ratio of Cracks to length	RM average percentage
11931	Top	26	0	------	0	10.0%
10932	Top	30	0	------	0	8.0%
10966	Top	22	0	------	0	6.5%
11922	Middle	34	0	------	0	10.0%
10929	Middle	29	0	------	0	6.5%
10961	Middle	28	0	------	0	8.5%
11921	Bottom	20	0	------	0	12.0%
11846	Bottom	42	0	------	0	7.5%
11105	Bottom	25	0	------	0	6.0%

Analysis

Samples located on the top shelf had a RM range of 10.5%-28.9% with a mean of 20.2% and a standard deviation of 5.3%. For grafts on the middle shelf they had a RM range of 11.3%-17.8% with a mean of 15.8% and a standard deviation of 2.4%. The grafts in the bottom shelf had a RM range of 6.8%-12.7% with a mean of 9.9% and a standard deviation of 1.9%.
The satellite samples are significantly different in RM from the whole cord while on the top and middle shelves, but they are similar when located on the bottom shelf.
When comparing the satellite samples from shelf to shelf they are not significantly different.
When comparing whole cord samples from shelf to shelf they are significantly different.
All 9 grafts with a RM range of 6.0%-12.0% (based on satellite samples) were crack-free.

Appendix D: Freeze Drying Variability

Discussion

The top shelf has a large range of residual moisture values and a higher mean RM. The middle shelf has a smaller range and a slightly smaller RM value. The bottom shelf has the smallest range and the lowest RM value. This makes sense since tissue can only get so dry, as the tissue reaches a maximal dryness it becomes more uniform throughout. So the smaller range is seen on the tissue with the smallest mean RM value.

Satellite samples do compare with each other from shelf to shelf but fall outside the range found on a whole cord for their shelves. In addition the satellite samples for the cord checked for cracks also fall outside the RM range found for the whole cord for each shelf.

Cords from shelf to shelf have decreasing RM values from the top shelf to the bottom, which can not be compared to each other. In other words, the shelves are drying differently and in turn producing different RM values.

Grafts with RM values of 6.0%-12.0% (satellite samples) were found to be without cracks. No samples, whether full length graft or satellite, were found to have RM below 3.6%, but the full-length graft did have RM values upwards of 28.9%.

Grafts in this study were not expected to have cracks because their RM values were estimated to be around 5% based on the time the freeze-dryer was stopped. According to RM-0001, cracks are observed 50% of the time when RM values range from 0.5% - 5%.

Conclusions

There is large variability within a single freeze-dryer with regards to its ability to evenly dry material. There is a 10.1% difference from the mean value of the top shelf (20.2%) and the mean value of the bottom shelf (9.9%). Statistics also confirm the significance of the different RM values among the three shelves. The satellite samples do not directly reflect the RM value of a whole vascular graft. Even though nine gr afts within a range of 6.0-12.0% were found to be without cracks the variability is very high

Appendix E: Biocompatibility Testing

Pyrogenicity:

Test System

A previously established test system designed to detect the presence of chemical pyrogens in solid materials was employed. In this model a test article is extracted in saline and the resulting solution is injected intravenously in New Zealand White Rabbits. The Rabbits are monitored for 3 hours for any febrile response to the injection. A febrile response is defined as an increase in temperature = 0.5°C.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of graft leachables to elicit a pyrogenic response was evaluated.

Each tissue sample was extracted @ 37°C for 24 hr in 4g tissue/80ml 0.9% saline and then 10ml extract per kg body weight of the animal was injected into each of three rabbits rabbit.

Results

The data are summarized in Table 1 and demonstrate that the UVG is not pyrogenic.

Table 1: Summary data of pyrogenicity testing.

DNase Soak Time (hours)	UVG Lot #	Maximum Remperature Rise (°C)		
		Animal		
		1	2	3
18	SFH-4	0.0	0.0	0.1
18	SFH-15	0.0	0.0	0.0
18	SFH-20	0.0	0.0	0.4
24	SFH-3	0.2	0.0	0.0
24	SFH-6	0.0	0.0	0.0
24	SFH-12	0.0	0.0	0.3

Appendix E: Biocompatibility Testing

Cytotoxicity:

Test System

A previously established test system designed to determine the cytotoxicity of solids and extracts was employed. The test consists of a confluent monolayer culture of L929 mouse fibroblasts. The culture is maintained in the presence of the test article for an additional 48 hours at 37°C and evaluated under a microscope biological reactivity using cytochemical stains. A sample is considered cytotoxic if it incites more than a mild response.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of the cytotoxicity of the graft and its leachables was evaluated.

Two different test articles were used; MEM elution for leachables, and agar diffusion to assess "direct contact". The MEM elution test samples were derived from extracting 1cm2 section of tissue in MEM @ 37°C for 24 hr in 10ml. Agar diffusion test samples were 1cm^2 sections of tissue placed directly against the 0.5-2g/ml agar layer covering the monolayer culture. Each test was performed in triplicate.

Results

The data are summarized in Table 2 and demonstrate that the UVG is not cytotoxic as reactivity ratings were zero for all samples.

Appendix E: Biocompatibility Testing

Table 2: Summary data of cytotoxicity testing.

DNase Soak Time (hours)	UVG Lot #	Agar diffusion			MEM elution		
		Plate			Plate		
		1	2	3	1	2	3
18	SFH-4	0	0	0	0	0	0
18	SFH-15	0	0	0	0	0	0
18	SFH-20	0	0	0	0	0	0
24	SFH-3	0	0	0	0	0	0
24	SFH-6	0	0	0	0	0	0
24	SFH-12	0	0	0	0	0	0

Hemolysis:

Test System

A previously established test system designed to determine the hemolytic potential of solids and extracts was employed. The test consists of a concentrate of human red blood cells in 0.9% saline. The concentrate is incubated in the presence of the test article for a 1 hour at 37°C. After incubation the optical density of the concentrate is evaluated using a spectrophotometer to assess the degree of cell lyses.

Test articles

Samples of umbilical vein graft were processed from six individual lots. The two worst case scenarios for potentially harmful residuals were tested in these studies; 24 hours detergent solution incubation (the maximum allowable time), followed by PBS rinses and either 18 or 24 hours (minimum and maximum allowable times respectively) of DNase treatment. These scenarios allow for the most detergent possible and either the most DNase or the least time for the DNase solution to act as a rinse of the detergent solution. The grafts sized to 16-18cm (approximately 4g rehydrated wet weight) packaged in Tyvek bags, sealed, and frozen. The samples were then thawed and rehydrated and the ability of the cytotoxicity of the graft and its leachables was evaluated.

Two different test articles were used; extracted samples for leachables, and intact tissue ta assess direct contact. The extraction test samples were derived from extracting 0.1g of tissue @ 37°C for 24 hr in 1.5ml of 0.9% saline. Direct contact test samples were 0.1g of tissue immersed directly in the red blood cell concentrate. Each test was performed in triplicate. Maximum acceptable levels of hemolysis were established as 5%.

Results

The data are summarized in Table 3 and demonstrate that the frozen UVG is not hemolytic as the percent hemolysis did not even approach the 5% level.

Appendix E: Biocompatibility Testing

Table 3: Summary data of cytotoxicity testing.

DNase Soak Time (hours)	UVG Lot #	Percent Hemolysis (%)					
		Direct contact			Extraction		
		Tube			Tube		
		1	2	3	1	2	3
18	SFH-4	0.39	0.08	0.70	0.00	0.00	0.47
18	SFH-15	0.08	0.78	0.16	0.00	0.08	0.00
18	SFH-20	0.00	0.00	0.00	0.00	0.00	0.47
24	SFH-3	0.39	0.31	0.08	0.08	0.00	0.00
24	SFH-6	0.00	0.00	0.00	0.00	0.00	0.00
24	SFH-12	0.00	0.00	0.00	0.00	0.00	0.00

Appendix F: Quality Plan for Vascular Grafts

Donor Screening

As specified by AATB all donors are assessed for risk of infectious diseases using a Donor Medical History & Behavioral Risk Assessment Questionnaire and a physical evaluation. The following serological testing is performed: HIV I&II, Hepatitis B&C, HTLV I&II, and Syphilis, all of which must be negative or the tissue is rejected for processing. A pathologist does a direct visual review of all umbilical cords before they are shipped to LifeCell. Tissue is released and approved for processing by LifeCell's medical director following review of all donor information.

Microbiological testing

All umbilical cords have 0.5cm samples cut before immersion in antibiotic solutions and sent for microbiological testing with enumeration. Tissue that is found to be positive for spore forming microbials is excluded from processing.

All lots have a 0.5cm sample taken that will be destructively tested in its entirety for sterility (14 day) in at least 300ml of solution as has been determined to be required for normal growth in Bacteriostatic-Fungistatic testing. Any positive growth results in graft rejection.

Processing

In order to significantly reduce the tissue bio-burden and minimize chance of infection, the tissue is exposed to various antibiotics covering a broad spectrum of activity and an antifungal agent. Following procurement, the tissue is shipped and stored in a solution containing antibiotics. The tissue is not in this solution for more than 30 hours. During processing at Lifecell the tissue is exposed to solutions that contain antibiotics and an antifungal agent.

Contamination and Cross contamination

All measures in place for the prevention of contamination and cross contamination are delineated in a standard operating procedure. The procedure requires that tissues from different donors may not be combined during processing. It prescribes that reusable instruments, equipment and supplies must be disinfected, cleaned, and sterilized between uses. Also, tissue is only exposed in controlled Class 100 areas and only approved disinfectants may be used as cleaning agents for those areas. Finally, the procedure directs additional standard procedures for biohazard waste disposal and management should that be needed.

Appendix F: Quality Plan for Vascular Grafts

A class 100 clean room is used to perform the tissue dissection. The qualification of this processing environment has been completed and is maintained in a controlled state using standardized procedures for operation, environmental monitoring and routine cleaning, hygiene and gowning. The area is annually certified.

All other processes involving tissue exposure are carried out in LifeCell's main cleanroom area and subject to the controls currently in place that define cleanroom hygiene, gowning, and cleaning and cleanroom environmental monitoring for those production areas.

Quality control testing

Grafts that are produces need to have some testing provided to provide an assurance that the vessels have been processed appropriately. The tests and pass/fail criteria chosen to be used for quality testing on every lot of vascular graft were developed and are described below.

The process calls for the removal of MHC I&II in the detergent incubation. The effectiveness will be tested using immunohistochemistry (IHC) to look for MHC in cryosections. As these are antigenic components of the cells, these tests also serve a safety function. Although the amount of MHC removal that is critical is not known, the experience with the process to date has been that removal is achieved. Therefore, a process drift will be noted by positive presence of MHC, and consequently a positive MHC stain will not be acceptable.

The process also calls for the substantial removal of the nuclear material often left after the detergent step, using a DNase solution. The presence of nuclear material has been evaluated using hematoxylin & eosin staining. Removal of DNA is not functionally critical as non-decellularized allografts are used clinically however, the experience with the process to date has been that removal is substantially achieved and its removal is believed to be beneficial. Therefore, a process drift will be noted by presence of significant cell associated hematoxylin staining and will constitute a failure.

Qualitative assessment of the matrix and basement membrane will also be performed by the project leader as an extra check that there has not been extreme damage to either from unexpected causes.

Appendix F: In Vivo Assessment of Umbilical Artery Grafts

SUMMARY REPORT: *IN VIVO* ASSESSMENT OF UMBILICAL ARTERY GRAFT IN A PORCINE MODEL-FEASIBILITY STUDY

EXECUTIVE SUMMARY

The objective of this study was to evaluate human umbilical arteries for applications suited to a small diameter graft. Umbilical arteries were evaluated for the capability to withstand a high pressure, high flow situation for a relatively short period of time (7 days), as well as to assess its resistance to occlusion and aneurysmal dilatation.

Two de-cellularized and vitrified human umbilical artery grafts, obtained from two distinct umbilical cords, were implanted bilaterally as common carotid interposition grafts in one pig. The umbilical arteries handled like well-behaved biological grafts with no gross bleeding or degeneration. The animal appeared to be doing well until day six when the pig was found dead by the animal caretaker.

The necropsy revealed that one of the vitrified human umbilical arteries ruptured; the other underwent a less catastrophic failure that resulted in a hemodynamic leak. Both grafts appeared patent, and neither graft became dilated.

OBJECTIVE

Acellular umbilical cord veins have been developed for use as vascular shunts, but the vein's diameter limits the applications. Methods for producing acellular vascular shunts using umbilical cord arteries will dramatically expand the application of vascular shunts. For example, the umbilical cord arteries are much more suitable for bypass surgery in patients with coronary artery disease: a disease that affects approximately 12 million people in the United States.

RESULTS

Two decellularized and vitrified human umbilical artery grafts, obtained from two distinct umbilical cords, were implanted bilaterally as common carotid interposition grafts in one pig. The grafts were thawed in PBS (pH 7.4) immediately prior to surgery, and photographed with a filled lumen (Figure 1).

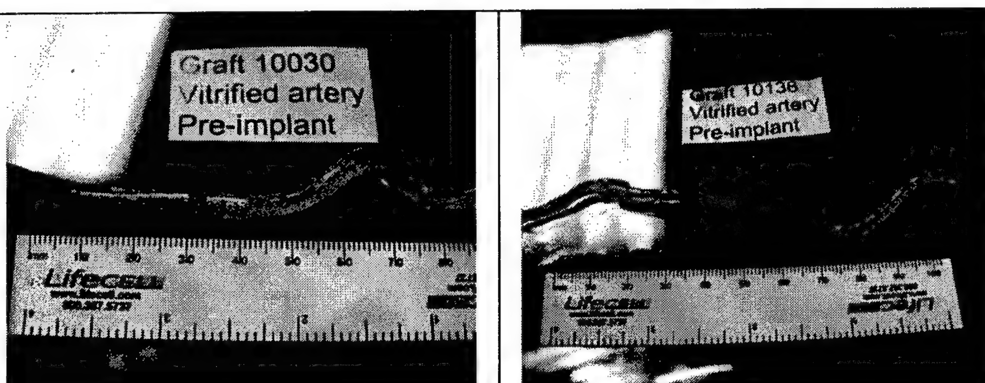


Figure 1: Photographs of umbilical arteries prior to implantation.

Appendix F: In Vivo Assessment of Umbilical Artery Grafts

Umbilical cord artery 10030A-1 was grafted to the pig's left carotid artery, end-to-side, to generate both the proximal and distal anastomosis, while artery 1036A-1 was similarly grafted to the pig's right carotid artery (Figure 2).

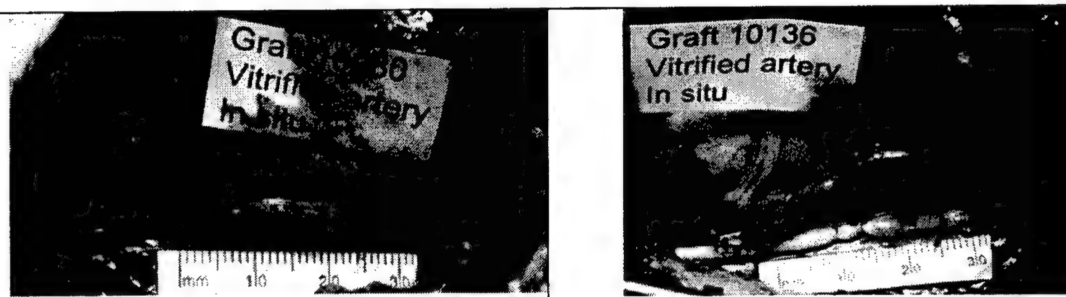


Figure 2: Umbilical artery immediately after implantation as carotid interpositional grafts in a porcine model.

Flow rates were measured at two times following surgery. The left carotid graft flow rate initially measured 40 ml/min, and sixty minutes later measured 200 ml/min. The right carotid graft flow rate was 35 ml/min initially, and at 85 ml/min thirty minutes later.

The pig was observed daily and appeared well with no complication until day 6 when the animal died shortly before noon. Examination of the animal revealed that both sides of the neck were swollen (Figure 3)

Figure 3: Gross photo of swollen neck at implant site post-mortem

Necropsy revealed large bilateral hematomas. Rupture clearly occurred of the umbilical artery 10136A-1 above the proximal anastomosis of the right carotid artery graft. Graft tissue remained attached to the anastomosis--indicating that the failure was not the anastomosis itself. No gross rupture of the umbilical artery 10030A-1 grafted to the left carotid artery occurred, but a large hematoma was evident (Figure 4).

Appendix F: In Vivo Assessment of Umbilical Artery Grafts

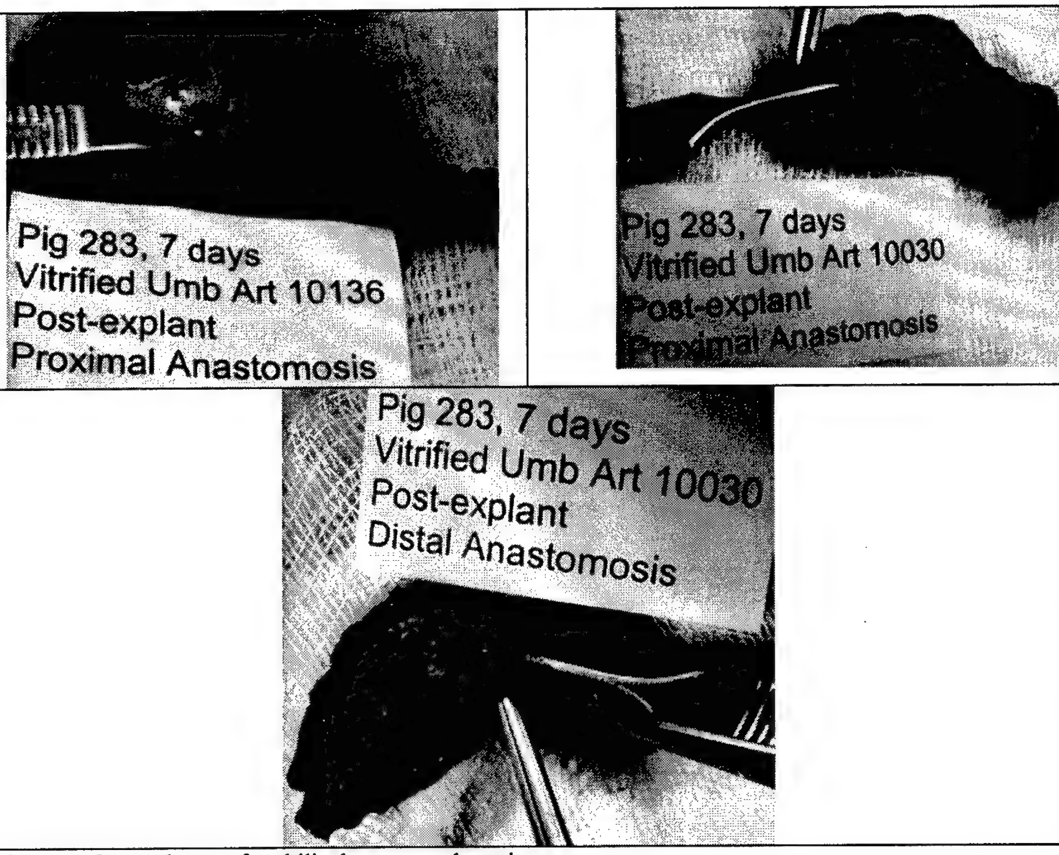


Figure 4: Gross photos of umbilical artery explantation

Gas bubbles next to the left carotid graft were noted (Figure 5). An infectious agent could be the source of the gas bubbles, however, both grafts had negative culture results prior to implant.

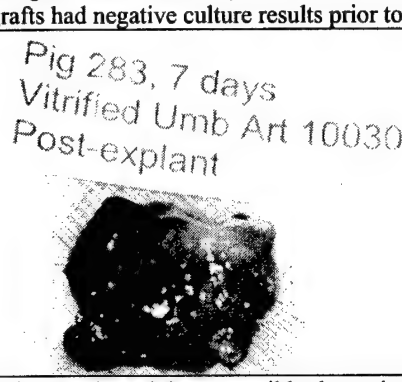


Figure 5: Gas bubbles documented on explanted tissue possibly due to infection.

Both grafts contained loose unorganized clot, and a probe easily passed into each graft. This supports that both grafts were patent. Additionally, no dilation was evident.

CONCLUSION

It is not clear what cause the failure of the grafts in this study. More rigorous evaluation of umbilical artery tissue is warranted.

Appendix G: Evaluation of Umbilical Artery Tissue Dissection

Objective

Observations about arteries that shred, break, and leak.

Data/Discussion

Veins from the umbilical cords were dissected in the tissue lab and used for experimentation. The arteries were further dissected from the amnion using the blunt dissection method. The table below documents the ability to recover acceptable lengths or arterial tissue. Arteries that have blood clots or blood related staining of the tissue are noted. Breakage and leaking of the grafts either during dissection or filling the graft after dissection are recorded as well. There is a full photographic record. Figure 1 shows an example of pre-dissection umbilical arteries with adherent tissue and dissected umbilical arteries. Table 1 catalogues the % of breaks and leaks in unstained sections of artery and stained sections of artery.

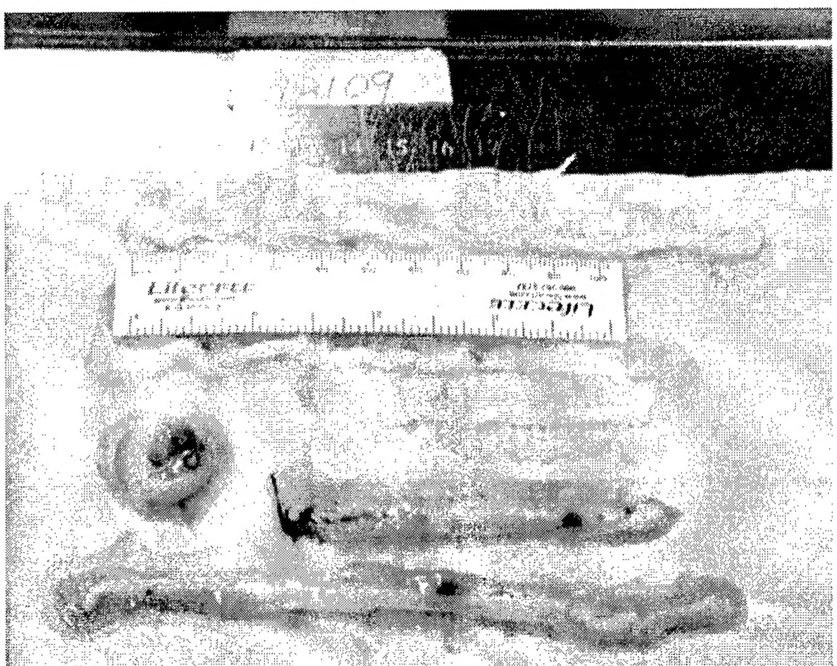


Figure 1: Example of pre-dissection (bottom) umbilical arteries with adherent tissue and following dissection (top).

Example:
12018 A_1 had two breaks total. One occurred at a clot, one in a stained area, and both breaks involved clean areas, therefore, one break occurred at a clot in a clean area, and the other occurred at the interface of a clean and stained area. 12018 A_2 had three breaks total: two at clots (actually between the four clots) and one in a clean area.

Appendix G: Evaluation of Umbilical Artery Tissue Dissection

		Clots		Unstained Portions		Stained Portions		Kinks		Total
Cord #	Cord length	#	break/ Leak	%	Break/ leak	%	break/ leak	#	break/leak	break/ leak
11972 A1	42 cm	0	0	5%C	0	95%C	0	0	0	**0**
11972 A2		0	0	40%V	0	35%V/25 %C	0	0	0	**0**
11984 A1	30 cm	0	0	10% C	0	90% C	0	1	1	**1**
11984 A2		0	0	10% C	0	90% C	0		1	**1**
11810 A1	48 cm	2	1	39%C	1	61%C	0	0	0	**1**
11810 A2		2	0	30%C	0	70% C	0	1	0	**0**
11811 A1	55 cm	TNTC	5	0%	0	100% C	5	0	0	**5**
11811 A2		0	0	0%	0	100% C	TNTC last 20 cm	1	0	**TNTC**
12040 A1	38 cm	0	0	50%V	0	50%V	0	0	0	**0**
12040 A2		0	0	83%C	2	17%C	1	0	0	**2**
12017 A1	57 cm	TNTC	TNTC			100% C	TNTC	0	0	**TNTC**
12017 A2		TNTC	TNTC			100% C	TNTC	0	0	**TNTC**
11948 A1	33 cm	10	2	3%C	1	97%C	2	0	0	**2**
11948 A2		TNTC	2	14%V	1	86%V	2	0	0	**3**
11971 A1	48 cm	TNTC	TNTC			100% C	TNTC		TNTC	**TNTC**
11971 A2		TNTC	TNTC			100% C	TNTC		TNTC	**TNTC**
12018 A1	36 cm	1	1	20%C 40%V	2	40%V	1	0	0	**2**
12018 A2		4	2	50%V	1	50%V		0	0	**3**
12109 A1*	16 cm	1		100%C	1			0	1	**1**
12109 A2*		1		100%C	1			0	1	**1**

Appendix G: Evaluation of Umbilical Artery Tissue Dissection

Analysis

- Arteries tend to shred upon dissection in areas where they appear dried out, collapsed, and difficult to see due to lack of blood. 10 breaks were counted in the clean areas. Of these, 5 were in clean stretches, 3 were at clots in the middle of clean stretches and 2 were at the interface where clean areas met stained areas.

- Arteries that are extremely clotted, with clots too numerous to count, also tend to shred at multiple points along the vessel. Six arteries with clots too numerous to count correspondingly tore, broke or shred frequently during dissection. Seven arteries with 18 clots sustained breaks at the clots in 6 instances. As mentioned above, three were located in clean areas, and three of those were between clots that were very close together.

- Any large anatomical disturbance, like a "sausage link" kink in the cord, or an unusual twist, has greater potential to break at that point. In one such instance, a cord had looped back on itself. One artery passed through the loop but broke at dissection. The other traveled around the loop, came off in one piece, but later leaked at that point when perfused. Similarly, an extremely bulgy looking clot – wider than 3 mm – may have greater potential to break at that point just before or after the clot. In one instance, breaks occurred on either side of a very large (4mm diameter) clot.

- Finally, arteries that were stained, but not clotty, seemed to hold together. Five arteries with at least 50% consistent staining, but no clots, did not break. Two others with a variegated pattern, but at least 50% stained, also did not break.

Although not currently standard procedure, arteries were perfused with PBS after dissection to check for leaks. There were three instances were arteries that came off in one piece and looked clean would leak, while variegated arteries held the fluid. **It does not seem at this point that it is a simple matter to judge the competence of the artery by color or successful dissection.**

A fresh 15cm length of cord #12109 was dissected to note the differences. The blood was a brighter red and flowed more freely in contrast to frozen/thawed arteries. The arteries looked fairly clean with just a few clots. However, while both 15 cm+ lengths of artery came off the vein in one piece, both broke upon further dissection. Afterward, the blood flushed easily from the artery's lumen when perfused with PBS, and didn't leave a stain, except for a small spot where a clot had been. These pieces did not leak when perfused. Perhaps the fact that the red blood cells had not been lysed by freezing the staining components, Hemoglobin and/or Heme, remained in the red blood cell. See figure 1, which shows dissected, flushed arteries at top, arteries in the amnion pedicle at the bottom, and a cross-section of whole cord at left.

Of the ten cords dissected and the potential for 20 grafts. After breakage the only long vessels left were 6 grafts varying in length from 28 cm to 41 cm but they varied in coloration. Only one 15 cm stretch was really clean a yield of about 2% based on length assessment. These cords were all received from NDRI. It is anecdotal that the cords received from SFH are typically less bloody and clotty than the cords received from Saint Francis Hospital.

Appendix G: Evaluation of Umbilical Artery Tissue Dissection

Conclusions

Fresh or frozen/thawed arteries did not demonstrate much difference in their ability to hold together during dissection. However, the ease of perfusing the fresh artery and the clean state achieved afterward warrants further investigation into this process. It may be feasible to flush the lumens of the artery at the first holding step stage. If so, this may yield a cleaner arterial graft later.